Kidney International, Vol. 63 (2003), pp. 1240–1248

Cux-1 transgenic mice develop glomerulosclerosis and interstitial fibrosis

Jennifer G. Brantley, Madhulika Sharma, Neal I. Alcalay, and Gregory B. Vanden Heuvel

Department of Anatomy and Cell Biology and University of Kansas Medical Center, Kansas City, Kansas

Cux-1 transgenic mice develop glomerulosclerosis and interstitial fibrosis.

Background. *Cux-1* is a murine homeobox gene that is highly expressed in the nephrogenic zone of the developing kidney. Transgenic mice ectopically expressing *Cux-1* develop renal hyperplasia associated with down-regulation of the cyclin kinase inhibitor p27. Because the reduction of p27 has been associated with mesangial cell proliferation and glomerular disease, we evaluated glomerular changes in Cux-1 transgenic mice.

Methods. Adult kidneys from Cux-1 transgenic mice were analyzed morphologically for changes in glomerular cell number and for changes in mesangial and interstitial extracellular matrix deposition. Mesangial matrix expansion was identified by light microscopy. Glomerular cell number was performed following immunohistochemistry. Type IV collagen deposition was analyzed by immunofluoresence and Western blotting. Renal function was evaluated by serum protein, blood urea nitrogen (BUN), creatinine, and electrolyte analysis, and by urine protein and creatinine analysis.

Results. In adult transgenic glomeruli, Cux-1 was ectopically expressed in mesangial cells, and this was associated with an increase in mesangial cell number, resulting from an increase in proliferation. There was a marked increase in mesangial matrix area in transgenic mice compared to non-transgenic littermates, related to an increase in type IV collagen. Podocyte foot process effacement was observed in transgenic mice, and this was related to an increase in urinary albumin. Interstitial fibrosis was also observed in transgenic kidneys.

Conclusion. These observations indicate that increased expression of Cux-1 in mesangial cells results in cell proliferation and mesangial expansion. In addition, these changes are potentially related to disruption of podocyte architecture leading to loss of filtration. These results suggest that expression of Cux-1 is sufficient to induce the early events of mesangioproliferative glomerulonephritis.

Key words: homeobox, cyclin kinase inhibitor, p27, mesangial cell hyperplasia.

Received for publication January 17, 2002
and in revised form September 5, 2002
Accepted for publication November 27, 2002

Cell proliferation is a hallmark of many glomerular diseases, including glomerulonephritis resulting from immunoglobulin A (IgA) nephropathy, lupus, mesangial proliferative glomerulonephritis, and diabetes [1–4]. Studies have shown that mesangial cell proliferation both precedes and predicts the onset of glomerulosclerosis [5–6]. In addition, in tubulointerstitial disease, early tubular and interstitial cell proliferation is associated with later development of fibrosis [7].

Cell proliferation is controlled by a group of nuclear proteins known as cell-cycle regulatory proteins. These proteins can positively [cyclins and cyclin-dependent kinases (CDK)] or negatively (cyclin kinase inhibitors) regulate cell proliferation. The cyclins form an active complex with a catalytic subunit, the CDK [8]. Both are located in the nucleus and specific cyclin-CDK complexes are expressed in each phase of the cell cycle. These complexes phosphorylate the protein retinoblastoma (RB), causing the release of the transcription factor E2F, which targets genes that are involved in DNA synthesis. Thus, cyclin-CDKs act through RB to induce DNA synthesis via E2F, driving the progression of the cell cycle [9]. Cyclin kinase inhibitors (CKI) are nuclear proteins that bind to the cyclin-CDK complex preventing the phosphorylation of RB and other substrates [10–11]. This prevents E2F release and subsequent gene activation dependent on E2F, resulting in cell-cycle arrest.

Cux-1 is the murine homolog of the *Drosophila* gene cut. Mammalian Cut homologs function as transcriptional repressors of genes specifying terminal differentiation in multiple cell lineages [12–20]. Upon terminal differentiation, Cut proteins are down-regulated or lose the ability to bind to the promoters, and transcription of the target genes is permitted. Recently, a role for mammalian Cut proteins in regulating cell proliferation has emerged. Cut proteins repressed the expression of the CKI, p21, during G_1-S phase [20]. In addition to their repressor function, mammalian Cut proteins have been implicated as transcriptional activators of histone gene expression, independent of E2F during G_1-S transition [21].

Transgenic mice constitutively expressing the homeobox gene *Cux-1* develop hyperplasia of several organs in which the transgene is ectopically expressed [22]. During normal kidney development, *Cux-1* expression is highly and transiently expressed, with highest expression restricted to the nephrogenic zone [23]. In the developing kidneys of transgenic mice, *Cux-1* is ectopically expressed in more highly differentiated glomeruli and tubules, and this is associated with reduced expression of the CKI p27. Transient transfection experiments demonstrated that Cux-1 is an inhibitor of *p27* gene expression [22]. Because down-regulation of p27 has been associated with the development of mesangioproliferative glomerular diseases in the kidney [6], we evaluated changes in glomeruli and in renal function in the *CMV/Cux-1* mice.

METHODS

Generation of mice and genotyping

Three independent lines of transgenic mice carrying the *CMV/Cux-1* transgene in the C57Bl/6 X C3H background were previously generated [22]. Adult transgenic mice were genotyped using Southern blot analysis as previously described [22]. Gender- and age-matched nontransgenic littermates were used as wild-type control mice in all experiments.

Immunohistochemistry

Isolated metanephroi were immersion fixed in 4% paraformaldehyde and blocked in paraffin. Tissue sections (5 μm thick) were deparaffinized with xylene and rehydrated with graded ethanols. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 minutes and the samples were then rinsed in phosphate-buffered saline (PBS). To obtain adequate signal, the slides were treated with antigen unmasking solution (Vector, Burlingame, CA, USA) according to manufacturer's protocol, or slides were treated for 10 minutes with trypsin (1 mg/mL) at 37°C. To reduce background, the sections were blocked for 1 hour at room temperature in 10% normal serum from the species the secondary antibody was made in. Antibody dilutions were 1:50 for CCAAT displacement protein (CDP) antibodies (Ab), 1:100 for desmin Ab, 1:100 for Wilm's tumor-1 (WT-1) Ab, 1:20 for platelet/endothelial cell adhesion molecule (PECAM) Ab, 1:100 for type IV collagen Ab, in 2% blocking serum in PBS. Slides were incubated at room temperature with 100 μL of antibody in a humid chamber and then washed four times in PBS. Biotin-conjugated secondary antibodies (Vector) were diluted 1:400 in PBS containing 2% serum from the species in which the secondary antibody was generated. After incubation for 1 hour at room temperature, slides were washed four times in PBS, then incubated with avidin-biotin-peroxidase complex (ABC-Elite; Vector) and then 3,3′-diaminobenzidine. The tissue sections were then dehydrated with graded ethanols and mounted with Permount (Fisher, USA). In order to identify the extent of mesangial matrix expansion, we stained wild-type and transgenic metanephroi with periodic acid-Schiff (PAS) stain. The extent of glomerulosclerosis was analyzed according to Raij, Azar, and Keane [24]. Glomerulosclerosis was defined by the presence of increased amounts of PAS-positive material within the glomeruli. The initial 100 glomerular profiles from four adult transgenic (two male and two female) and four gender- and age-matched wild-type mice were counted and the relative PAS-positive material was scored. For mesangial and podocyte cell counts, a minimum of 100 glomeruli from four adult transgenic (two male and two female) and four gender- and age-matched wild-type mice were scored. The number of mesangial cells and podocytes in wild-type or transgenic glomeruli were compared by using Student *t* test for paired samples. For analysis of interstitial fibrosis, kidney sections were stained with Masson-trichrome stain. Images were captured on a Leica DMR microscope (Leica, Wetzlar, Germany) equipped with an Optronics Magnafire digital camera (Optronics, Goleta, CA, USA). All images are representative of at least five from each of four wild-type or four transgenic kidneys.

Immunofluorescence

Metanephroi were isolated from 10% formalin-perfused mice and blocked in paraffin. Tissue sections (5 μm thick) were deparaffinized with xylene and rehydrated with graded ethanols. To block endogenous auto fluorescence sections were incubated with 1 mol/L NH_4Cl for 30 minutes. Sections were washed in phosphate buffered saline containing 0.1% Tween (PBST), blocked in 10% bovine serum albumin (BSA) at room temperature for 1 hour, and incubated with antibodies diluted in PBS with 3% BSA for 1 hour at room temperature. Antibody dilutions were 1:50 for CDP Ab, 1:20 for desmin Ab, 1:3000 for PCNA Ab, 1:100 for type IV collagen Ab. Sections were washed with PBST and incubated with fluorescein isothiocyanate (FITC) or Texas Red conjugated secondary antibodies (Vector). Sections were then washed, mounted with Vectashield (Vector), and viewed with a fluorescence microscope. Images were captured with an Optronics Magnafire digital camera. All images are representative of at least five from each of four wild-type or four transgenic kidneys.

Antibodies

Commercial reagents used were goat anti-CDP (Cux-1) (Santa Cruz #sc-6327; Santa Cruz Biotechnologies, Santa Cruz, CA, USA); rabbit antidesmin (Sigma #D-8281; Sigma Chemical Company, St. Louis, MO, USA); rabbit anti-WT-1 (Santa Cruz #sc-192); goat anti-PECAM (Santa Cruz #sc-1506); goat anti-type IV collagen-UNLB (unla-

beled) (Southern Biotechnology Associates, Inc., #1340-01, Birmingham, AL, USA); mouse anti-PCNA (Sigma #P-8825).

Western blot analysis

Whole 6-month-old kidney lysates (50 μg) were solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed on 4% to 15% gradient polyacrylamide gels, and transferred to nitrocellulose filters as described previously [22]. The immunoblot was blocked in 5% nonfat dry milk in PBST (PBS containing 0.1% Tween 20) for 1 hour at room temperature. Goat anti-type IV collagen (Southern Biotechnology Associates, Inc., #1340-01) antibody was added at a dilution of 1:100. After overnight at 4°C, filters were washed three times at room temperature with PBST, and incubated at room temperature for 1 hour with peroxidase-conjugated antigoat IgG (1:10000 dilution; Sigma). Following three additional washes with PBST, bound antibody was detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce) according to manufacturer's instructions, followed by exposure to x-ray film for 1 minute.

In some experiments, 2 μL and 6 μL of urine, collected from 6-month-old transgenic and nontransgenic mice housed in Nalgene metabolic cages for 24 hours, were electrophoresed with 20 μg of BSA on 4% to 20% polyacrylamide gels, and transferred to nitrocellulose filters. Sheep antialbumin antibody (AbCam #ab8940) was added at a dilution of 1:200, and bound antibody was detected as described above.

Electron microscopy

Following perfusion with 10% formalin, kidneys were collected and fixed in ice cold 2.5% gluteraldehyde in 0.1 mol/L phosphate buffer for 1 hour at 4°C, rinsed with phosphate buffer, and post-fixed with 1% osmium tetroxide for 1 hour. Tissues were then dehydrated through a series of ethanols followed by propylene oxide and embedded in EMBed 812 (Electron Microscopy Sciences, Fort Washington, PA, USA). Sections (1 μ thick) were stained with 1% toluidine blue and observed for regions that contained several glomeruli. Ultrathin sections (80 nm) were stained with urinyl acetate and lead citrate and viewed with a JOEL 100 CXII electron microscope. Images are representative of two wild-type and two transgenic kidneys.

Serum and urine chemistry

Urine and blood (200 to 500 μL) were collected prior to sacrifice. The mice were anesthetized with carbon dioxide then killed by cervical dislocation. Serum urea nitrogen, creatinine, total protein and electrolytes, and urine creatinine, protein, and total protein/creatinine ratios were determined using an autoanalyzer (Physicians Reference Laboratory, LLC, Overland Park, KS, USA). Urine creatinine, protein, and total protein/creatinine ratios were compared by using Student *t* test for paired samples.

RESULTS

In our previous work, we characterized changes in kidney development in transgenic mice expressing the wild-type *Cux-1* gene under the control of the CMV immediate early gene promoter. In the developing kidney, ectopic expression of Cux-1 in capillary loop-staged and maturing glomeruli resulted in down-regulation of the CKI p27 [22]. *CMV/Cux-1* mice developed hyperplasia in tissues in which the transgene was ectopically expressed, similar to p27 null mice [25–27]. Because decreased expression of p27 is associated with mesangial cell proliferation, we have now evaluated changes in adult glomeruli in *CMV/Cux-1* mice. Adult kidneys from *CMV/Cux-1* mice ectopically express Cux-1 in both glomerular and tubular cells (Fig. 1). Cux-1 was highly and ectopically expressed in a subset of cells in glomeruli from transgenic mice, but not in the glomeruli of nontransgenic littermates (Fig. 1 C and D). Transgenic glomeruli double-stained with Cux-1 and desmin, to identify mesangial cells, revealed that Cux-1 was ectopically expressed in mesangial cells (Fig. 1E). In our previous work, we had observed a 23% increase in glomerular cell number in transgenic mice [22]. To determine which cells were increased in transgenic glomeruli, we stained adult kidneys sections with desmin, WT-1 to identify podocytes, and PECAM to identify endothelial cells (Fig. 2). Podocyte and mesangial cell counts were then performed on a minimum of 100 glomeruli from 6-month-old transgenic and nontransgenic mice. These results, shown in Table 1, indicated that the increased cells in glomeruli were primarily mesangial cells. In contrast, there was no significant increase in podocytes. We were unable to accurately count PECAM-positive cells in glomerular sections. However, no obvious differences in PECAM staining were observed (Fig. 2 E and F). We had previously shown an increase in cell proliferation in both glomeruli and tubules of Cux-1 transgenic mice. To determine the identity of the proliferating cells in transgenic glomeruli, we performed double-labeling experiments in which we stained transgenic and nontransgenic glomeruli for desmin and PCNA (Fig. 3). This revealed that most of the proliferating cells in the glomerulus are mesangial cells.

In addition to an increase in cellularity, PAS staining of transgenic and wild-type kidneys revealed extensive expansion of the mesangial matrix in the transgenic glomeruli, compared to non-transgenic glomeruli (Fig. 4). To quantitate the extent of mesangial increase, the first 100 glomeruli from four transgenic and wild-type 1-year-

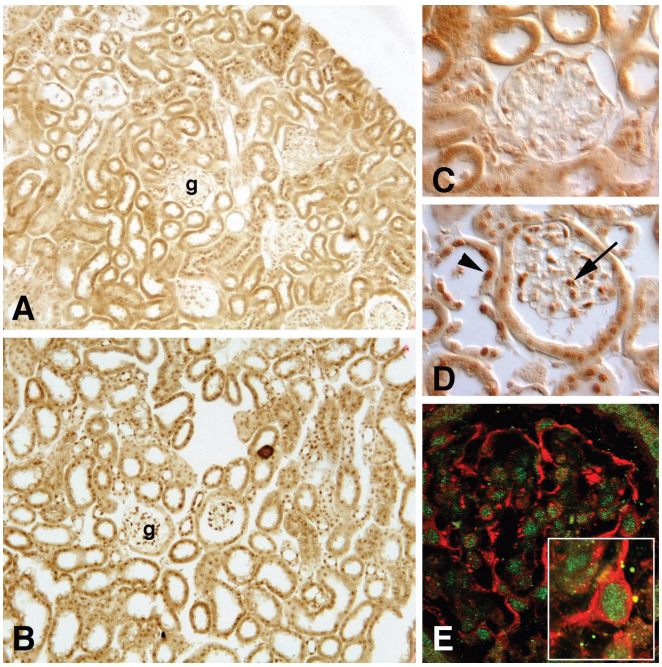

Fig. 1. Expression of Cux-1 protein in adult transgenic kidney. Light micrographs of wild-type (*A* and *C*) and transgenic (*B* and *D*) adult kidneys. High levels of Cux-1 protein are detected in the nuclei of glomeruli (g) and tubules of adult transgenic mice (A and B). Cux-1 is minimally expressed in glomeruli (g) or tubules of age-matched wild-type kidneys (A). Higher magnification reveals high levels of nuclear Cux-1 expression in a subset of cells in transgenic glomeruli, while Cux-1 is minimally expressed in the glomeruli of nontransgenic mice (C and D). Double labeling of transgenic kidney sections with Cux-1, in green, and desmin, in red, (*E*) reveals that Cux-1 is ectopically expressed in mesangial cells. Inset in (E) shows nuclear localization of Cux-1 in a cell labeled for desmin. Original magnification, (A) and (B) ×200; (C), (D), (E) ×400; inset to (E) ×630.

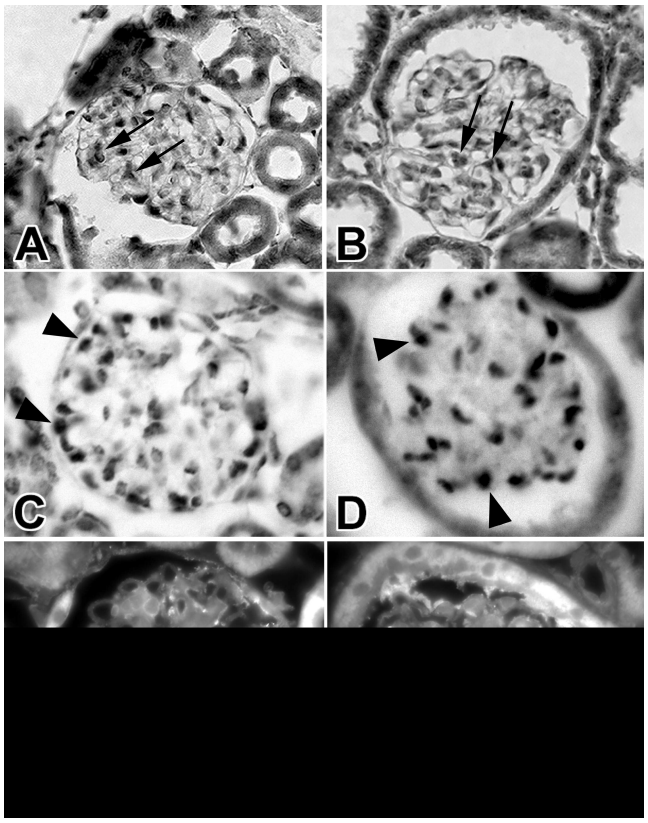

Fig. 2. Antibody labeling of glomerular cells in *CMV/Cux-1* transgenic kidneys. Antidesmin staining, indicated byarrows, was used to quantitate mesangial cells in wild-type *(A)* and transgenic *(B)* glomeruli. Desmin positive cell counts reveal that mesangial cell number is increased in transgenic glomeruli (see Table 1). Anti-Wilm's tumor (WT)-1 staining, indicated by arrowheads, was used to quantitate podocytes in wild-type *(C)* and transgenic *(D)* glomeruli. WT-1–positive cell counts indicate that podocytes are not increased in transgenic glomeruli (see Table 1). Anti-PECAM labeling outlines endothelial cells in wild-type *(E)* and transgenic *(F)* glomeruli. No obvious differences in PECAM staining were observed between 100 wild-type and transgenic glomeruli. Original magnification, ×1000.

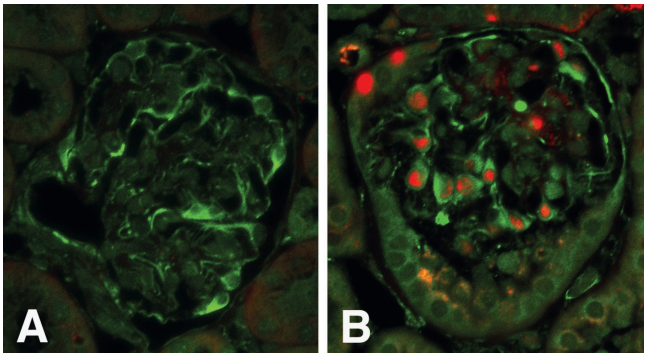

Fig. 3. Increased proliferation of mesangial cells in transgenic kidneys. (*A*) Wild-type glomerulus. (*B*) Transgenic glomerulus. Proliferating cell-nuclear antigen (PCNA) staining (red) is observed in transgenic glomeruli, but not wild-type glomeruli. Desmin staining (green) reveals that many of the proliferating cells are mesangial cells. Original magnification, ×630.

Table 1. Glomerular cell counts

	Wild-type	Transgenic	Relative increase
Podocytes	30 (2.0)	28 (1.3)	0%
Mesangial cells	33 (2.9)	41 (2.3)[a]	24%

Cell counts were obtained from 5 μm sections of age-matched adult wild-type and transgenic kidney stained with anti-Wilm's tumor (WT-1) antibody to detect podocytes, and antidesmin antibody to detect mesangial cells. A minimum of 100 glomeruli from four adult transgenic (two male and two female) and four gender and age-matched wild-type mice were scored. Data are expressed as group means of the number of positive cells per glomerulus. Standard error is indicated in parentheses.

[a] $P = 0.0006$

old kidneys were scored according to the following scale: (*1*) 0% to 25% PAS-positive material in the glomerulus, (*2*) 26% to 50% PAS-positive material in the glomerulus, (*3*) 51% to 75% PAS-positive material in glo-

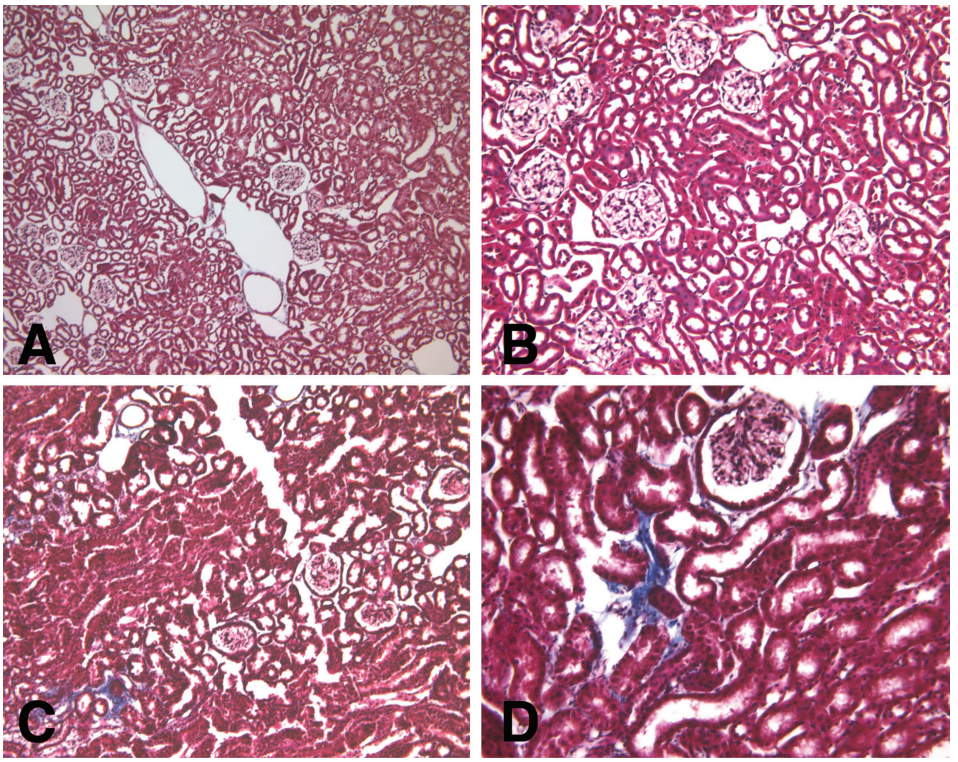

Fig. 6. Interstitial fibrosis in Cux-1 transgenic kidneys. Masson-trichrome staining of wild-type (*A* and *B*) and transgenic (*C* and *D*) kidney sections showing interstitial fibrosis in peritubular spaces of transgenic kidneys. Original magnification, (A) and (C) ×50; (B) ×100; (D) ×200.

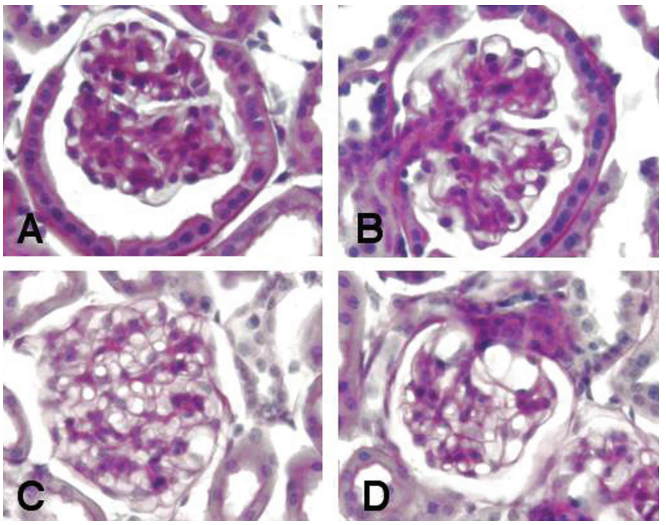

Fig. 4. Mesangial increase in transgenic kidneys. Periodic acid-Schiff (PAS)–stained sections of transgenic (*A* and *B*) and wild-type (*C* and *D*) kidneys demonstrating matrix expansion in cytomegalovirus (CMV)/Cux-1 mice. The first 100 glomeruli from wild-type and transgenic kidney sections were scored for the percent PAS-positive material in each glomerulus (see Table 2). Representative glomeruli were scored as follows: (A) 51% to 75% PAS positive; (B) 26% to 50% positive; (C) and (D) 0% to 25% PAS positive. Original magnification, ×1000.

Table 2. Quantitation of mesangial increase

	I	II	III	IV
Wild-type	95	5	0	0
Transgenic	46	39	14	1

Glomeruli from wild-type and transgenic kidneys were scored for the percent periodic acid-Schiff (PAS) positive material in the glomerulus. A score of I corresponds to 0% to 25% PAS-positive material; II, 26% to 50% PAS-positive material; III, 51% to 75% PAS-positive material; and IV, 76% to 100% PAS-positive material. Shown are the average of the first 100 glomeruli scored from four adult transgenic (two male and two female) and four gender-matched wild-type mice. Characteristic staining is shown in Figure 5.

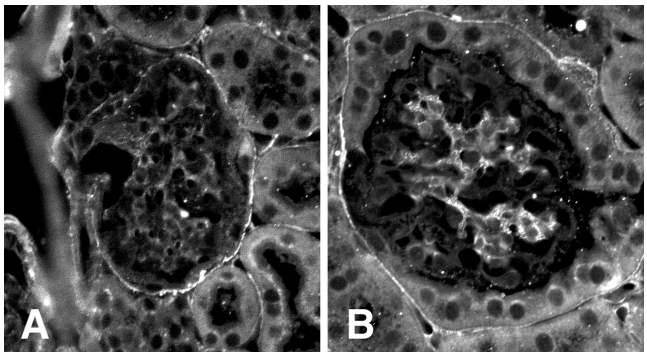

Fig. 5. Localization of type IV collagen in transgenic glomeruli. *(A)* Wild type glomerulus. *(B)* Transgenic glomerulus. Type IV collagen staining in the mesangial matrix is increased in transgenic glomeruli. Original magnification, ×400.

merulus, and (*4*) 76% to 100% PAS-positive material in glomerulus. These results indicated that more than half of the transgenic glomeruli were sclerotic (Table 2). The mesangial sclerosis varied between individual glomeruli but was regularly distributed between cortical and juxtamedullary regions.

To further characterize the increase in mesangial matrix in transgenic glomeruli, we stained transgenic and

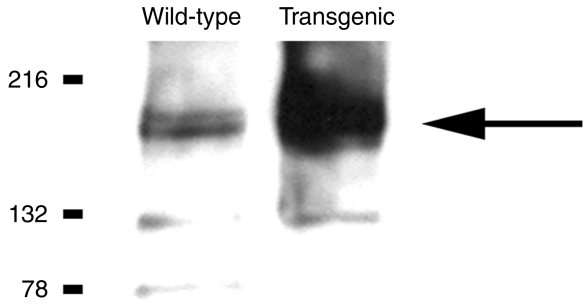

Fig. 7. Western blot of type IV collagen. Fifty micrograms of total kidney lysate isolated from wild-type and transgenic 6-month-old kidneys was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The arrow indicates an increase in type IV collagen in transgenic kidneys. Positions of molecular weight standards (×kD) are shown on right.

nontransgenic kidney sections for changes in extracellular matrix synthesis. At 6 months of age, transgenic glomeruli exhibited an increase in type IV collagen deposition within the mesangium, compared to nontransgenic age-matched littermates (Fig. 5). In addition to glomerulosclerosis, Masson-trichrome staining revealed interstitial fibrosis in the kidneys of Cux-1 transgenic mice, but not in nontransgenic littermates (Fig. 6). To determine the increase in type IV collagen synthesis, Western blot analysis was performed on total kidneys homogenates isolated from transgenic and nontransgenic littermates. Figure 7 shows that type IV collagen was markedly increased in transgenic kidneys compared to wild-type kidneys.

Previously, we showed that kidney/body weight ratio and heart/body weight ratio were increased in *CMV/Cux-1* mice beginning at 6 weeks of age. To evaluate physiologic changes in *CMV/Cux-1* mice, we measured plasma levels of sodium, potassium, chloride, creatinine, blood urea nitrogen, and total protein at 6 and 12 months of age. No significant differences were observed between transgenic and *CMV/Cux-1* mice (Table 3). However, a significant increase in urinary protein was observed between Cux-1 transgenic and nontransgenic mice beginning at 6 months of age (Table 4). Western blot analysis of urine from transgenic and nontransgenic showed that albumin was the major protein component in the urine of transgenic mice (Fig. 8). To determine the basis for the albuminuria in transgenic mice, we examined transgenic and nontransgenic glomeruli by transmission electron microscopy. Six-month-old Cux-1 transgenic mice showed evidence of podocyte foot process effacement, while age-matched nontransgenic mice exhibited normal podocyte foot processes (Fig. 9).

DISCUSSION

In our previous study we generated transgenic mice constitutively expressing the homeobox gene *Cux-1* using the CMV immediate early gene promoter. Two independent transgenic lines ectopically expressed Cux-1 protein in multiple tissues, and this was associated with hyperplasia where the transgene was expressed. In the kidney, we observed a pronounced increase in cellularity in both tubules and glomeruli. In addition, we observed that proximal tubule epithelial cells continued to proliferate in the adult kidney, displacing the parietal epithelium [22]. Finally, we showed that Cux-1 is a transcriptional regulator of the cell cycle, negatively regulating the expression of the CKI p27.

In the present study, we have characterized the glomerular changes in the Cux-1 transgenic mice. To determine which cells were increased in transgenic glomeruli, we used markers for three different cell types: WT-1, for podocytes, desmin for mesangial cells, and PECAM for endothelial cells. We were able to quantitate mesangial cells and podocytes, and these results show that mesangial cells are increased in transgenic glomeruli. The 24% increase in mesangial cell number correlated well with the overall 23% increase in glomerular cell number previously reported [22]. And while we were unable to accurately count endothelial cells, PECAM labeling of transgenic glomeruli was not markedly different than in wild-type glomeruli. There was no increase in podocyte cell number in the transgenic glomeruli. During glomerulogenesis in transgenic mice, Cux-1 is ectopically expressed in the endocapillary cells of capillary loop-staged glomeruli, but not in the overlying podocytes. In this current study we show that Cux-1 continues to be ectopically expressed in the glomeruli of adult transgenic kidneys, specifically in mesangial cells. Moreover, double staining for desmin and PCNA revealed that mesangial cells in adult glomeruli continue to proliferate. Thus, ectopic expression of Cux-1 in the developing and mature glomeruli results in mesangial cell proliferation contributing to the increase of mesangial cell number. In our previous studies, we showed that apoptosis during glomerular development was unchanged, indicating that the increase in cellularity results from an increase in cell proliferation, and not from a decrease in apoptosis [22].

Mammalian Cut protein expression or activity is restricted to proliferating cells in multiple tissues [28]. In the developing kidney, *Cux-1* is highly expressed in the nephrogenic zone in both mesenchymal cells (uninduced and condensed mesenchyme) and epithelial cells (ureteric buds, comma and S-shaped bodies). At later stages of nephrogenesis, *Cux-1* is down-regulated, coinciding with exit from the cell cycle and terminal differentiation [23]. Recent studies have demonstrated a role for mammalian cut proteins in cell-cycle regulation. First, Cux-1 was identified as the DNA binding component of the cell-cycle–regulated histone gene transcription factor HiNF-D [21]. Second, Cux-1 DNA binding activity was shown to fluctuate during the cell cycle with highest

Table 3. Serum concentrations of electrolytes, blood urea nitrogen (BUN), creatinine, and protein

	6-month-old control ($N = 3$)	6-month-old transgenic ($N = 12$)	12-month-old-control ($N = 8$)	12-month-old transgenic ($N = 15$)
Serum sodium concentration *meq/L*	154.7 (1.2)	157.4 (2.0)	154.4 (1.2)	153.6 (4.0)
Serum potassium concentration *meq/L*	9.9 (0.1)	9.1 (0.3)	9.4 (0.3)	9.3 (0.3)
Serum chloride concentration *meq/L*	114 (0.6)	111.3 (0.7)	113.1 (2.0)	110.2 (2.7)
Urea nitrogen concentration *mg/dL*	27.3 (1.8)	27.6 (1.5)	25.8 (1.9)	27.7 (1.2)
Serum creatinine concentration *mg/dL*	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)
Serum protein concentration *gm/dL*	6.9 (0.3)	6.4 (0.2)	8.5 (1.3)	6.6 (0.2)

Standard error is indicated in parentheses.

Table 4. Urine protein, creatinine, and protein/creatinine ratios

	6-month-old control ($N = 10$)	6-month-old transgenic ($N = 16$)	12-month-old-control ($N = 9$)	12-month-old transgenic ($N = 10$)
Urine protein concentration *mg/dL*	88.0 (35.4)	604.4 (134.6)[a]	157.8 (67.5)	696.9 (143.5)[d]
Urine creatinine concentration *mg/dL*	69.6 (6.7)	50.9 (3.7)[b]	71.6 (7.9)	51.4 (3.7)[e]
Urine total protein/creatinine ratio	1.7 (0.8)	12.6 (3.1)[c]	2.3 (0.9)	14.5 (3.4)[f]

Standard error is indicated in parentheses. [a] $P = 0.007$; [b] $P = 0.015$; [c] $P = 0.012$; [d] $P = 0.005$; [e] $P = 0.029$; [f] $P = 0.004$

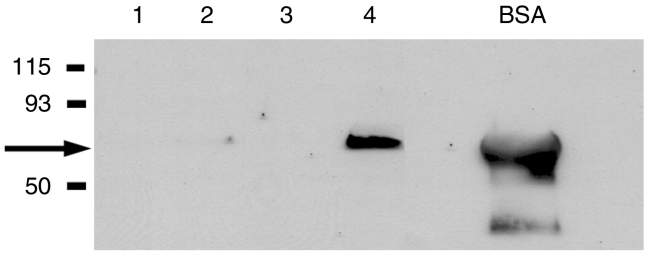

Fig. 8. Albuminuria in Cux-1 transgenic mice. Two and six microliters of urine from wild-type (lanes 1 and 2) and transgenic (lanes 3 and 4) mice, respectively, was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The presence of albumin in the urine was detected using a sheep polyclonal antibody to albumin. Twenty micrograms of bovine serum albumin (BSA) was added as a control.

activity during late G_1 and S phase [20, 29, 30]. Third, several of the targets of Cux-1 repression are cell-cycle–regulated, including the CKI p21 [20, 29, 30]. In addition to p21, we have recently identified the CKI p27 as another target of Cux-1 repression [22]. The pattern of p27 expression is inverse to Cux-1 in the developing kidney, suggesting that Cux-1 functions to repress *p27* gene expression during the early stages of nephrogenesis [31]. Moreover, ectopic expression of Cux-1 in maturing glomeruli of transgenic mice results in aberrant down-regulation of p27[22]. And *CMV/Cux-1* transgenic mice develop hyperplasia in organs in which the transgene is ectopically expressed. This is similar to p27 null mice, which exhibit hyperplasia of all organs studied [25–27].

In recent years, the role of cell-cycle regulation during normal kidney development and renal disease processes has been the focus of intense examination. These studies have demonstrated a central role for the CKIs p21 and p27 in the regulation of cell proliferation in kidney disease (reviewed in [2, 3]). In experimental mesangial proliferative glomerulonephritis, the onset of mesangial cell proliferation was associated with a reduction in the levels of p27 [6]. Furthermore, at the time of maximal mesangial cell proliferation, p27 levels were undetectable. To further explicate the role of p27 in renal disease, experimental glomerulonephritis was induced in p27 knockout and p27 wild-type mice [32]. Renal function was substantially decreased in nephritic p27 −/− mice compared to controls, and this was associated with increased glomerular cell proliferation, apoptosis, and matrix protein expansion. When unilateral ureteral obstruction was applied to p27 −/− mice, tubular epithelial cell proliferation increased twofold compared to obstructed wild-type tubular cell proliferation [32]. These results suggest that p27 protects against inflammatory injury in the kidney. Similarly, when experimental glomerulonephritis was induced in p21 null mice, there was a fourfold increase in glomerular cell proliferation, with a significant decrease in renal function, compared to nephritic control mice [33]. And when unilateral ureteral obstruction was applied to p21 −/− mice, interstitial cell proliferation increased twofold compared to obstructed wild-type [34]. However, no difference in tubular cell proliferation was observed.

CMV/Cux-1 mice develop glomerulosclerosis that is evident at 6 months of age. Extracellular matrix components accumulate in the mesangium, with a specific increase in type IV collagen. The mechanisms by which glomerular lesions are generated in *CMV/Cux-1* mice appear are not clear at present. Mesangial cell proliferation is an important component of many forms of glomerular disease [1, 35]. Mesangial cell proliferation precedes the accumulation of extracellular matrix that is charac-

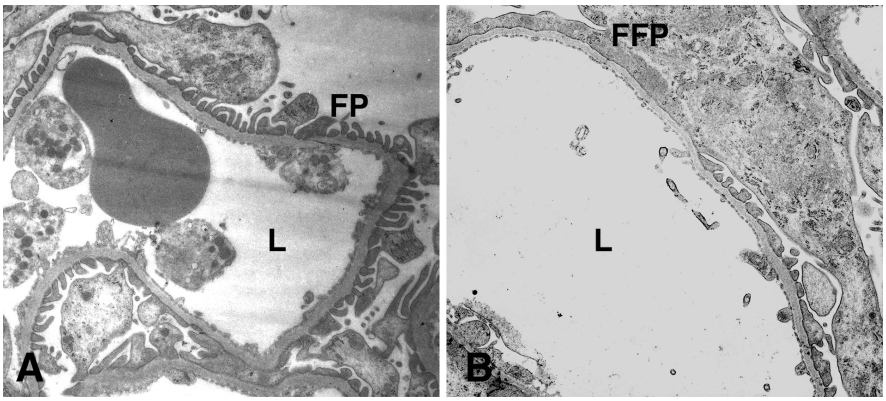

Fig. 9. Electron micrographs of glomeruli. Wild-type (*A*) and transgenic (*B*) glomeruli are shown at medium (×14,400) magnification. The glomerular basement membrane appears normal, but many foot processes (FP) are fused (FFP) in transgenic mice. L is capillary lumen.

teristic of glomerulosclerosis [36]. Moreover, blocking mesangial cell proliferation at the level of the cell cycle prevents the accumulation of extracellular matrix in the Thy-1.1 model of experimental glomerulonephritis [37]. However, while experimental glomerulonephritis induced in p27 null mice results in substantially decreased renal function compared to controls, untreated p27 null mice do not develop glomerulosclerosis [32]. Therefore, while the overall increase in mesangial cells correlates with the down-regulation of p27, there are likely other factors contributing to the development of renal disease in the Cux-1 transgenic mice. One possible explanation is repression of p21 in mesangial cells by ectopically expressed Cux-1. In contrast to p27, the role of p21 is more complex. Recent studies have shown that p21 can function in the assembly of cdk4/cyclin D complexes, in addition to its role as an inhibitor of CDK/cyclin complexes [38]. While the levels of p27 are high in normal glomeruli, the levels of p21 are low [6]. In the anti-Thy-1.1 model of experimental glomerulonephritis, the resolution of mesangial cell proliferation is associated both with a return to baseline levels of p27 and an increase in p21 expression [6]. This increased level of p21 expression remains following the resolution of proliferation, leading to the speculation that p21 has a long-term role in resolving mesangioproliferative disease [4]. Thus, ectopic expression of Cux-1 in adult mesangial cells might prevent this increase in p21. This would lead to continued mesangial cell proliferation, and accompanied with mesangial matrix synthesis, which contribute to the development of glomerular disease. This is consistent with the increase in PCNA positive mesangial cells observed in the Cux-1 transgenic mice.

CMV/Cux-1 mice exhibited proteinuria evident at 6 months of age. In addition, urine creatinine levels were reduced resulting in increased protein/creatinine ratios. Increased protein/creatinine ratios have been observed in other transgenic mouse models that develop glomerulosclerosis [39]. Ultrastructural analysis of transgenic kidneys revealed abnormalities in the visceral epithelial cells (podocytes) lining the glomerular basement membrane. While podocytes from nontransgenic kidneys showed normal podocyte foot processes, the transgenic mice exhibited foot process effacement. Podocyte foot process effacement is observed in a number of glomerular diseases associated with proteinuria [40]. While we did not observe an increase in podocyte number, podocyte foot process effacement is associated with increased mechanical stress [40]. One possibility is that the increased number of mesangial cells, together with mesangial matrix expansion, results in increased stress on the podocytes leading to a change in the podocyte phenotype.

We also observed interstitial fibrosis in the transgenic kidneys, as determined by Masson-trichrome staining. A correlation of renal function with interstitial fibrosis, as compared to glomerulosclerosis, in human disease has been identified [41–43]. Moreover, human endothelin-1 transgenic mice develop both glomerulosclerosis and interstitial fibrosis and exhibit reduced kidney function [44]. In contrast, human endothelin-2 transgenic rats develop glomerulosclerosis without interstitial fibrosis and do not exhibit reduced renal function [45]. With the exception of proteinuria, renal function is not compromised in the Cux-1 transgenic mice. Thus, the functional significance of the interstitial fibrosis observed in the Cux-1 transgenic mice is unclear.

CONCLUSION

In summary, ectopic expression of Cux-1 in adult kidneys results in the development of glomerulosclerosis, interstitial fibrosis, and proteinuria. Thus, these animals provide an interesting new experimental model to study the mechanisms of Cux-1–induced mesangial cell proliferation.

ACKNOWLEDGMENTS

We thank Rosetta Barkley, Gabor Kemeny, and Eileen Roach for expert technical assistance. We thank Dr. Dale Abrahamson and Dr. James Calvet for many helpful discussions. This work was supported by the American Heart Association (G.B.V.H.), and by NIH grant DK-53877A (G.B.V.H.).

Reprint requests to Gregory B. Vanden Heuvel, Ph.D., Department of Anatomy and Cell Biology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160
E-mail: gvandenheuvel@kumc.edu

REFERENCES

1. Klahr S, Schreiner G, Ichikawa I: The progression of renal disease. *N Engl J Med* 318:1657–1666, 1988
2. Shankland SJ: Cell-cycle control and renal disease. *Kidney Int* 52:294–308, 1997
3. Shankland SJ, Wolf G: Cell cycle regulatory proteins in renal disease: Role in hypertrophy, proliferation, and apoptosis. *Am J Physiol* 278:F515–F529, 2000
4. Schocklmann HO, Lang S, Sterzel RB: Regulation of mesangial cell proliferation. *Kidney Int* 56:1199–1207, 1999
5. Floege J, Burns MW, Alpers CE, *et al*: Glomerular cell proliferation and PDGF expression precede glomerulosclerosis in the remnant kidney model. *Kidney Int* 41:297–309, 1992
6. Shankland SJ, Hugo C, Coats SR, *et al*: Changes in cell-cycle protein expression during experimental mesangial proliferative glomerulonephritis. *Kidney Int* 50:1230–1239, 1996
7. Kliem V, Johnson RJ, Alpers CE, *et al*: Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. *Kidney Int* 49:666–678, 1996
8. Morgan DO: Principles of CDK regulation. *Nature* 374:131–134, 1995
9. Nevins JR: E2F: A link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 258:424–429, 1992
10. Sherr CJ, Roberts JM: Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev* 9:1149–1163, 1995
11. Peter M, Herskowitz I: Joining the complex: cyclin-dependent kinase inhibitory proteins and the cell cycle. *Cell* 79:181–184, 1994
12. Superti-Furga G, Barberis A, Schreiber E, Busslinger M: The protein CDP, but not CP1, footprints on the CCAAT region of the gamma-globin gene in unfractionated B-cell extracts. *Biochim Biophys Acta* 1007:237–242, 1989
13. Skalnik DG, Strauss EC, Orkin SH: CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. *J Biol Chem* 266:16736–16744, 1991
14. Andres V, Nadal-Ginard B, Mahdavi V: *Clox*, a mammalian homeobox gene related to *Drosophila* cut, encodes DNA-binding regulatory proteins differentially expressed during development. *Development* 116:321–334, 1992
15. Valarche I, Tissier-Seta JP, Hirsch MR, *et al*: The mouse homeodomain protein Phox2 regulates Ncam promoter activity in concert with Cux/CDP and is a putative determinant of neurotransmitter phenotype. *Development* 119:881–896, 1993
16. Dufort D, Nepveu A: The human cut homeodomain protein represses transcription from the c-myc promoter. *Mol Cell Biol* 14:4251–4257, 1994
17. Banan M, Rojas IC, Lee WH, *et al*: Interaction of the nuclear matrix-associated region (MAR)-binding proteins, SATB1 and CDP/Cux, with a MAR element (L2a) in an upstream regulatory region of the mouse CD8a gene. *J Biol Chem* 272:18440–18452, 1997
18. Higgy NA, Tarnasky HA, Valarche I, *et al*: Cux/CDP homeodomain protein binds to an enhancer in the rat c-mos locus and represses its activity. *Biochim Biophys Acta* 1351:313–324, 1997
19. Liu J, Bramblett D, Zhu Q, *et al*: The matrix attachment region-binding protein SATB1 participates in negative regulation of tissue-specific gene expression. *Mol Cell Biol* 17:5275–5287, 1997
20. Coqueret O, Berube G, Nepveu A: The mammalian Cut homeodomain protein functions as a cell-cycle-dependent transcriptional repressor which downmodulates p21WAF1/CIP1/SDI1 in S phase. *EMBO J* 17:4680–4694, 1998
21. van Wijnen AJ, van Gurp MF, de Ridder MC, *et al*: CDP/cut is the DNA-binding subunit of histone gene transcription factor HiNF-D: A mechanism for gene regulation at the G_1/S phase cell cycle transition point independent of transcription factor E2F. *Proc Natl Acad Sci USA* 93:11516–11521, 1996
22. Ledford, A.W., Brantley, J.G., Kemeny, *et al*: Deregulated expression of the homeobox gene Cux-1 in transgenic mice results in down regulation of $p27^{kip1}$ expression during nephrogenesis, glomerular abnormalities, and multiorgan hyperplasia. *Dev Biol* 245:157–171, 2002
23. Vanden Heuvel GB, Bodmer R, McConnell KR, *et al*: Expression of a cut-related homeobox gene in developing and polycystic mouse kidney. *Kidney Int* 50:453–461, 1996
24. Raij L, Azar S, Keane W: Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. *Kidney Int* 26:137–143, 1984
25. Fero ML, Rivkin M, Tasch M, *et al*: A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85:733–744, 1996
26. Kiyokawa H, Kineman RD, Manova-Todorova KO, *et al*: Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 85:721–732, 1996
27. Nakayama K, Ishida N, Shirane M, *et al*: Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85:707–720, 1996
28. Nepveu A: Role of the multifunctional CDP/Cut/Cux homeodomain transcription factor in regulating differentiation, cell growth and development. *Gene* 270:1–15, 2001
29. van Wijnen AJ, Cooper C, Odgren P, *et al*: Cell cycle-dependent modifications in activities of pRb-related tumor suppressors and proliferation-specific CDP/cut homeodomain factors in murine hematopoietic progenitor cells. *J Cell Biochem* 66:512–523, 1997
30. Aziz F, van Wijnen AJ, Stein JL, Stein GS: HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2-dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition. *J Cell Physiol* 177:453–464, 1998
31. Combs HL, Shankland SJ, Setzer SV, *et al*: Expression of the cyclin kinase inhibitor, p27kip1, in developing and mature human kidney. *Kidney Int* 53:892–896, 1998
32. Ophascharoensuk V, Fero ML, Hughes J, *et al*: The cyclin-dependent kinase inhibitor p27Kip1 safeguards against inflammatory injury. *Nat Med* 4:575–580, 1998
33. Kim YG, Alpers CE, Brugarolas J, *et al*: The cyclin kinase inhibitor p21CIP1/WAF1 limits glomerular epithelial cell proliferation in experimental glomerulonephritis. *Kidney Int* 55:2349–2361, 1999
34. Hughes J, Brown P, Shankland SJ: Cyclin kinase inhibitor p21CIP1/WAF1 limits interstitial cell proliferation following ureteric obstruction. *Am J Physiol* 277:F948–F956, 1999
35. Johnson RJ: The glomerular response to injury: Progression or resolution? *Kidney Int* 45:1769–1782, 1994
36. Pesce CM, Striker LJ, Peten E, *et al*: Glomerulosclerosis at both early and late stages is associated with increased cell turnover in mice transgenic for growth hormone. *Lab Invest* 65:601–605, 1991
37. Pippin JW, Qu Q, Meijer L, Shankland SJ: Direct in vivo inhibition of the nuclear cell cycle cascade in experimental mesangial proliferative glomerulonephritis with Roscovitine, a novel cyclin-dependent kinase antagonist. *J Clin Invest* 100:2512–2520, 1997
38. LaBaer J, Garrett MD, Stevenson LF, *et al*: New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 11:847–862, 1997
39. Doublier S, Seurin D, Fouqueray B, *et al*: Glomerulosclerosis in mice transgenic for human insulin-like growth factor-binding protein-1. *Kidney Int* 57:2299–2307, 2000
40. Shirato I: Podocyte process effacement in vivo. *Microsc Res Tech* 57(4):241–246, 2002
41. Nath KA: Tubulointerstitial changes as a major determinant in the progression of renal damage. *Am J Kidney Dis* 20:1–17, 1992
42. Nath KA: The tubulointerstitium in progressive renal disease. *Kidney Int* 54:992–994, 1998
43. Walls J: Relationship between proteinuria and progressive renal disease. *Am J Kidney Dis* 37(1 Suppl 2):S13–S16, 2001
44. Hocher B, Thone-Reineke C, Rohmeiss P, *et al*: Endothelin-1 transgenic mice develop glomerulosclerosis, interstitial fibrosis, and renal cysts but not hypertension. *J Clin Invest* 99:1380–1389, 1997
45. Hocher B, Liefeldt L, Thone-Reineke C, *et al*: Characterization of the renal phenotype of transgenic rats expressing the human endothelin-2 gene. *Hypertension* 28:196–201, 1996